Comment Submission
Federal Communication Commission
ET Docket Nos. 03–137 and 13–84; FCC 13–39

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Subject: Establishing rf exposure limits for children (NOI #53)

Ref: "Exposure to 900 MHz Electromagnetic Field Induces an Unbalanace Between Pro-Apoptotic and Pro-Survival Signals in T-Lymphoblastoid Leukemia CCRF-CEM Cells" (Marinelli, La Sala, *et al*; Journal of Cellular Physiology, 198: 324-332; 2004)

Comment:

While research has generally concluded that low-frequency electromagnetic field (EMF) exposure causes biological changes that can be linked to increased risk of cancer, there is not conclusive evidence regarding the health risks of high-frequency EMFs. This study examines the effect of high-frequency EMFs on in vitro cell survival. Leukemia cells were exposed to high frequency EMFs, at 900 MHz, aryi for vng lengths of time. T-lymphoblastoid leukemia cells were used because they are responsible for 80% of child acute leukemia, and there is evidence that they are particularly susceptible to damage from high frequency EMFs.

When the cells were exposed for 2-12 hours, they demonstrated increased DNA breaks and early activation of genes that induce apoptosis. Cells exposed for longer time periods (24-48 hours) activation of genes that control pro-survival signals. The results suggest that when cancer cells are initially exposed to EMF, they incur DNA damage. However, this damage invokes a self-defense response that, when the exposure continues, could actually allow the cells to survive and reproduce for a longer period of time. Any change in the balance between pro-apoptotic and pro-survival signals disrupts normal cell growth rate, and this study, along with many other similar experiments, provide strong evidence that EMFs can alter the expression of genes that control those signals.

JOURNAL OF CELLULAR PHYSIOLOGY 198:324–332 (2004)

Exposure to 900 MHz Electromagnetic Field Induces an Unbalance Between Pro-Apoptotic and Pro-Survival Signals in T-Lymphoblastoid Leukemia CCRF-CEM Cells

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It has been recently established that low-frequency electromagnetic field (EMFs) exposure induces biological changes and could be associated with increased incidence of cancer, while the issue remains unresolved as to whether high-frequency EMFs can have hazardous effect on health. Epidemiological studies on association between childhood cancers, particularly leukemia and brain cancer, and exposure to low- and high-frequency EMF suggested an etiological role of EMFs in inducing adverse health effects. To investigate whether exposure to high-frequency EMFs could affect in vitro cell survival, we cultured acute T-lymphoblastoid leukemia cells (CCRF-CEM) in the presence of unmodulated 900 MHz EMF, generated by a transverse electromagnetic (TEM) cell, at various exposure times. We evaluated the effects of high-frequency EMF on cell growth rate and apoptosis induction, by cell viability (MTT) test, FACS analysis and DNA ladder, and we investigated pro-apoptotic and pro-survival signaling pathways possibly involved as a function of exposure time by Western blot analysis. At short exposure times (2–12 h), unmodulated 900 MHz EMF induced DNA breaks and early activation of both p53-dependent and -independent apoptotic pathways while longer continuous exposure (24–48 h) determined silencing of pro-apoptotic signals and activation of genes involved in both intracellular (Bcl-2) and extracellular (Ras and Akt1) pro-survival signaling. Overall our results indicate that exposure to 900 MHz continuous wave, after inducing an early self-defense response triggered by DNA damage, could confer to the survivor CCRF-CEM cells a further advantage to survive and proliferate. J. Cell. Physiol. 198: 324–332, 2004. © 2003 Wiley-Liss, Inc.

Epidemiological studies on association between childhood cancers, particularly leukemia and brain cancer, and exposure to low- and high-frequency electromagnetic field (EMF) suggested an etiological role of EMFs in inducing adverse health effects (Wertheimer and Leeper, 1979; Savitz et al., 1988, 1995; Ahlbom et al., 1993, 2000; Theriault et al., 1994; Gurney et al., 1996; Preston-Martin et al., 1996). Although it has been well established that low-frequency EMFs exposure induces biological changes, including effects at both cytoplasmic membrane (Bersani et al., 1997) and nuclear levels (Jin et al., 1997) and an increase in the transcription level of specific genes (see for review, Goodman and Blank, 2002), the issue remains unresolved as to whether high-frequency EMFs can have hazardous effect on health. Few recent data suggested an effect of high-frequency EMF on cell proliferation (Kwee and Raskmark, 1999; Velizarov et al., 1999) as well as on activation of c-*jun* and c-*fos* oncogenes transcription (Rao and Henderson, 1996; Ivaschuk et al., 1997; Goswami et al., 1999). In vivo studies on blood lymphocytes of workers exposed to

Contract grant sponsor: ISPESL, PF-D1P1Ag-U025-2000; Contract grant sponsor: WWF-Italy; Contract grant sponsor: MURST-LAG-CO3; Contract grant sponsor: CNR-Italy.

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Received 6 March 2003; Accepted 21 July 2003

DOI: 10.1002/jcp.10425

microwaves radiation reported a considerable micronucleus incidence, a widely recognized hallmark of apoptosis, and significant increase in chromosomal aberrations similar to those observed on workers exposed to chemical pollutants (Garaj-Vhrovac et al., 1990; Maes et al., 1993, 1995). Furthermore, microwave radiation caused single- and double-strand DNA breaks in brain cells of rat both in vitro and in vivo (Lai and Singh, 1995, 1996; Adey, 1997) either immediately or 4 h after EMF radiation as well as in T-lymphoblastoid cells (Phillips et al., 1998) suggesting that high-frequency EMFs might act as DNA damage agent at cellular level.

Acute T-lymphoblastic leukemia (T-ALL) is responsible for 80% of childhood acute leukemia with a peak incidence occurring between 3 and 7 years of age. T-ALL also occurs in adults, where it comprises 20% of all adult leukemia. Some toxins such as benzene, some chemotherapeutic agents and radiation are thought to contribute to the induction of leukemia. Moreover, abnormalities in chromosomes may also play a role in the development of acute leukemia and it has been shown to be associated with aggressive nature of childhood T-ALL (O'Connor et al., 1991; Hoelzer et al., 2002). Both epidemiological and experimental data suggest that leukemic cells could be particularly susceptible to high-frequency EMFs exposure. Therefore, we investigated the effects of unmodulated 900 MHz EMF on the survival chance of a T-lymphoblastoid leukemia cell line (CCRF-CEM), as a function of exposure time.

Normal cell growth rate is the result of a balance between pro-apoptotic and pro-survival signals. Any unbalance in this equilibrium predisposes to transformation and, when the cell self-defense mechanisms fail, to cancer. Multiple interactions between cell cycle control proteins, mainly pRb/p105 and its related proteins, and pro-apoptotic p53-dependent and -independent pathways have been shown (Hsieh et al., 1999). *pRb/p105* is a tumor suppressor gene (Cinti and Giordano, 2000) recognized to be a central component of signaling pathways that negatively control cell proliferation. pRb/p105 mediates growth suppression functions by binding E2F transcription factors thus leading to the inhibition of E2Fs transactivation activity. E2F1 is the prototype member of E2F family and its target genes are involved not only in S-phase progression but also in G1-arrest and apoptosis. E2F1 has been shown to induce p53-dependent and -independent apoptosis since it can control accumulation of p53 (Hiebert et al., 1995; Kowalik et al., 1998), transcription of p73 (Irwin et al., 2000; Stiewe and Putzer, 2000; La Sala et al., 2003), and transcription of Apaf-1, a key element of apoptosome (Moroni et al., 2001). The *p53* tumor suppressor gene encodes a sequence specific transcription factor, which has anti-proliferative and pro-apoptotic effects and is stimulated in response to a variety of stress signals. p53 protein directly stimulates the expression of p21/WAF1, an inhibitor of cyclin-dependent kinases (CDKs), and bax, the best characterized mediators of p53-induced apoptosis (Carr, 2000). p53 is frequently mutated in a variety of tumors and previous studies have described two heterozygous p53 missense mutations (Cheng and Haas, 1990; Cinti et al., 2000) in CCRF-CEM cells, that do not impair its ability to bind DNA, as well as its transactivating activity (Park et al., 1994; Cinti et al., 2000). Moreover, cell survival is determined also by extracellular signals whose transduction depends on the activation of intracellular pathways. Ras protein is a GTPase protein bound to plasma membrane and is activated by a variety of external signals such as UV irradations, osmotic stresses and others (Macaluso et al., 2002). Ras-GTP activates a cascade of serine/threonine protein kinases leading to transcriptional activation of many genes involved in cell cycle progression (Gille and Downward, 1999). Ras has been shown to protect cells from apoptosis through activation of Akt1 via PI3-kinase, which provides a universal survival signal (Kennedy et al., 1997; Downward, 1998; Macaluso et al., 2002). Activated Akt1 (Akt1-P) promotes the decrease of transcription of pro-apoptotic genes and is implicated in the negative regulation of pRb/p105 functions (Khwaja et al., 1997; Du and Montminy, 1998). Activated Ras and/or Akt1 protect cells from apoptosis by preventing cytochrome c release and apoptosome formation, a common event in many forms of apoptosis (Rytomaa et al., 2000).

Here we show that short time exposure to unmodulated 900 MHz EMF induced in CCRF-CEM cells DNA breaks and early activation of both p53-dependent and -independent apoptotic pathways while at longer time of exposure we found an increase in DNA synthesis (S-phase) and the activation of pro-survival Ras pathways. These results strongly suggest that high-frequency EMFs affect cellular systems by inducing both genotoxic damage and changes in gene expression levels.

MATERIALS AND METHODS

Cell exposure system

To guarantee the best field homogeneity throughout the culture medium so that all the cells receive the same dose inside the culture flasks, we designed a transverse electromagnetic (TEM) cell (Fig. 1) able to be fed by frequencies greater than 800 MHz with characteristic impedance of 50 Ω. The unmodulated 900 MHz electromagnetic field was generated by a TEM cell placed inside an incubator NAPCO 9500-IR with infrared temperature control maintained at culture conditions 37°C, 95% humidity, and 5% CO_2. The TEM cell is a specially constructed copper box of 19, $25 \times 20 \times 50$ cm with 3 mm thickness in order to obtain about 50 Ω impedance, which is more suitable for the petri dishes. Its geometry and field propagation has been well described in literature (Stuchly and Stuchly, 1996). The box contains the "strip line," a flat copper septum which divides the inner

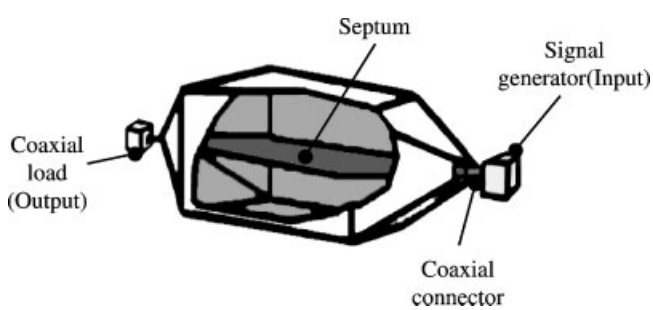

Fig. 1. Sketch of transverse electromagnetic (TEM) cell. TEM cell is a 3 mm copper box of 19, $25 \times 20 \times 50$ cm with a tapered horizontal copper septum which divides the inner space into two parts.

room into two equal parts. The tapering at the ends of septum, allows the mechanical matching between the strip line and the input and output 50 Ω standard N coaxial connectors. The cell is terminated into a 50 Ω load through a simple tuneable impedance matching section. The transformer-impedance adapter inserted between the TEM cell and the final load minimizes steady-state waves.

To allow gaseous exchanges between the incubator and the cell culture, one side of TEM wall was made of a removable 2.5 mm size copper mesh. The cultures were exposed to an EMF produced by a RF system sketched as a block diagram in Figure 2. In detail, we used a HP 8620C sweep oscillator as a signal generator covering the range 0.01–2.4 GHz via the HP86222B plug-in, a HP 796D directional coupler to feed a PM 1038 scalar meter equipped with CRT display necessary to optimise and continuously check the input return loss (RL) of the TEM cell. Ancillary equipment was a universal counter HP5316A and a power meter HP431A. Properly adjusted, the system measured 27 dB of RL at the 900 MHz frequency with and without the petri dish inside the TEM cell. This was the necessary condition favoring the best uniformity of the electric field with no differences higher than 2dB.

Microwave exposure

Nine hundred megacycles per second field was applied as continuous waves (CW) with 1 mW power input generating an electric field, inside the TEM cell and perpendicular to the septum plane, of 2.96 V/m and a magnetic field of 8 mA/m at a power density of 23.68 mW/m^2 resulting in a specific absorption rate (SAR) value of 3.5 mW/kg in the cell cultures. Fifty hertz electric and magnetic field generated by the incubator did not exceed 0.8 V/m and 0.16 μT, respectively all along the exposure times. The exposure times were 2, 4, 12, 24, 48 h.

Cell culture position

Five petri dishes of 10 cm diameter containing the cells cultured in 20 ml of medium were placed in the TEM cell at 1 cm distance above and below the septum.

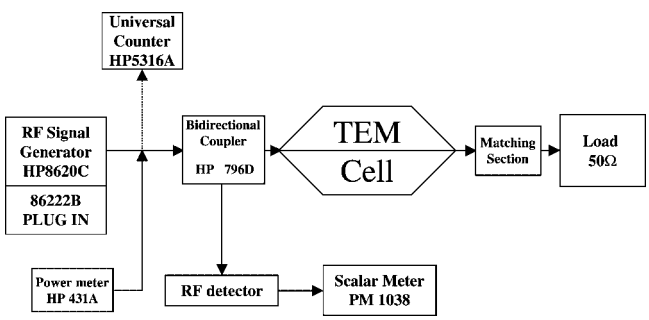

Fig. 2. Experimental cell-exposure arrangement. The electromagnetic signal is generated by signal generator, HP 8620C sweep oscillator covering the range 0.01–2.4 GHz via the HP86222B plug-in, which produce a field inside the TEM cell through the HP 796D directional coupler. The signal was output to the 50 Ω final load by a transformer-impedance adapter. In order to optimize and continuously check the input return loss (RL) inside the TEM cell a RF detector and PM 1038 scalar meter were connected. Universal counter HP5316A and a power meter HP431A were used as ancillary equipments.

The cultures did not exceed more than one third of the volume between the septum and the outer wall.

Administered field check

Spectrum analyzer equipped with a magnetic loop EMCO (1 cm diameter) or with electrospherical probe (3 cm diameter) were used in order to measure the magnetic and electric field generated by the system within the TEM cell. Detected values matched the theoretically expected values within the systematic instrumental range of errors.

Temperature control

In order to detect whether the administered power determined any thermal increment in the culture medium, a continuous temperature monitoring was performed in the medium and inside the petri dishes along the exposure time. To monitor the temperature, isolated thermo-couples and conventional alcohol thermometer were used. Thermo-probes were positioned inside the petri dishes placed in the upper plate of the TEM cell, inside and outside the TEM cell. During our performed experiments we detected no more than 0.15°C of temperature difference so that the observed EMF effects were independent from thermal phenomena.

Sham exposure

In order to check the effect of the complete equipment on the culture cells, CCRF-CEM cells were cultured for 2, 24, and 48 h inside and outside the TEM cell without electromagnetic field emission (generator switched off). At the end of the sham exposure, the cell viability test (MTT) and FACS analysis were carried out on cells inside TEM cell and compared to those outside the TEM (control).

Cell culture

Human CCRF-CEM obtained from the American Type Culture Collection (ATCC, Manassas, VA), were cultured in total volume of 20 ml RPMI-1640 medium supplemented with 10% FCS in petri dishes of 10 cm diameter at a density of 1×10^5 cells/ml and exposed to unmodulated electromagnetic fields of 900 MHz for 2, 4, 12, 24, and 48 h. Briefly, for each experiment, exponentially growing cells from a 175 cm^2 flask were scattered in the petri dishes and five petri dishes were located inside the TEM cell and the same quantity of cells was used as a control in the same incubator outside the TEM cell. The exposed and unexposed cells were consecutively picked up finely up to 48 h. As a further control system, the cells not exposed to fields were incubated in a different incubator from that containing the TEM cell. To have well-controlled biological experimental system, we performed the experiments at following condition: the diameter of the petri dishes were half of the strip line size, and the dishes were placed in the volume formed by the strip line and one half size of both upper and bottom distance of the septum from walls. The amount of medium was always of 20 ml/dish. The cells, which intially float in the medium, line down on the bottom of the petri disk in a few minutes. For this reason the attenuation of field at the bottom of the petri dish used can be ignored since it has been calculated to be less than

2% (Burkhardt et al., 1996). The medium was always pre-heated at 37°C in order to avoid the temperature raise time and the stress to the cells. All the experiments were performed comparing 900 MHz EMF-exposed with unexposed cells and, to avoid the variability inherent to the used assays, all tests were performed for fifteen independent experimental exposures. Any measure was performed soon after the end of the various exposure periods.

Cell viability test (MTT)

The MTT was performed following manufacturer's instructions (Cell Proliferation Kit I-MTT, Roche, Manheim, Germany). Hundred microliter/well of exposed and non-exposed cell suspension at 2, 4, 12, 24, and 48 h were aliquoted in a microtiter plate (tissue culture grade, 96-wells, flat bottom). Cells were incubated with MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, final concentration 0.5 mg/ml) for 4 h at 37°C. After this incubation period, purple formazan salts crystals were formed by a NADP/NADPH dependent process, from metabolically active cells. These salt crystals are insoluble in aqueous solution, but may be solubilized by adding the solubilization solution (0.01 M HCl, 10% SDS) and incubating the plates overnight in humidified atmosphere (37°C, 5% CO_2). The solubilized formazan product was spectrophotometrically (550–690 nm) quantified using an ELISA plate reader. The number of viable cells is directly correlated to the amount of purple formazan crystals formed.

Statistical analysis

Each control and exposed sample at 2, 4, 12, 24, and 48 h were aliquoted in twelve wells of a microtiter plate. The relative absorbance (550–690 nm) of each well was spectrophotometrically quantified and the mean values and standard deviation (SD) were calculated.

For each experimental setting, the cell viability index was calculated by making a ratio between the mean absorbance (Abs) values of the examined sample and a reference value (Abs value of cell suspension at 0 h). Statistical significance of the differences between controls and exposed cells was evaluated by Student's t-test.

Flow cytometry (FACS) analysis

In order to determine the percentage of cell population in different cell cycle phases, the cells, cultured for 2, 24, and 48 h, with or without electromagnetic field, were fixed in 70% ethyl alcohol at 4°C for 30 min. The nuclei were stained with 25 μg/ml of propidium iodide and incubated with 1 mg/ml of RNases for 1 h at 37°C. The nuclear DNA content which discriminate the cell cycle phases was determined using flow cytometry using Becton–Dickinson FACScan.

DNA ladder

A large quantities of CCRF-CEM cells (10^7), for each samples, was pelleted, washed in phosphate-buffer saline (PBS) and gently suspended in 500 μl of lysis buffer (1× PBS, 1% Nonidet P40, 0.5 ng/ml Proteinase K). During lysis, samples were kept on ice for 1 h. After incubation, the lysates were centrifuged at 14,000 rpm for 15 min. The supernatants were treated with RNAse A (100 μg/ml) at 37°C for 30 min. Four microliters of 6× gel loading solution (Sigma Chemical, St. Louis, MO) were added to 20 μl of each mixture and applied on 2% agarose gel. Gel electrophoresis (3 V/cm) was proceeded in 1× TBE buffer, then the gel was stained with ethidium bromide (1 μg/ml) water bath.

Western blot analysis

Whole cell lysates were prepared by re-suspending pelleted cells in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 10 mM disodium pyrophosphate, 10 g/ml aproptinin, 10 g/ml leupeptin). Forty grams of proteins were denatured by boiling in 2× sample buffer (100 mM Tris HCl pH 6.8, 200 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and size fractionated by electrophoresis in SDS/polyacrilamide gel and transferred onto a nitrocellulose membrane (BioRad, Hercules, CA). After saturation with 3% fat-free-milk and 2% BSA solution, the membranes were incubated with the following monoclonal antibodies: anti-human-bax (2D2) (Kamiya Biomedical Company, Seattle, WA), anti-Bcl2 (100/D5) (Kamiya Biomedical Company), anti-p53 (Ab6) (Calbiochem, Darmstadt, Germany), anti-p21 (4D10) (Medac Diagnostika, Wedel, Germany), anti-E2F1 (KH95) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-H-Ras (F235) (Santa Cruz Biotechnology). For pRb/p105, p73 Akt1 and phospho-Akt expression analysis the membranes were incubated polyclonal antibodies: anti-pRb antibody (C15), anti-p73 (H-79), anti-Akt1 (D-17) (Santa Cruz Biotechnology) and anti-phospho-Akt (Ser473) (New England BioLabs, Beverly, MA). To normalize Western blot analysis the anti-actin antibody (Sigma) was used. After three washings with PBS-Tween-20, the membranes were incubated with secondary anti-mouse, anti-goat, or anti-rabbit IgGs, coupled with horseradish peroxidase (Amersham, Life Science, Buckinghamshire, UK). Signal was detected using the ECL system (Amersham, Life Science, UK).

RESULTS

Effects of EMF on cell viability, cell growth rate, and apoptosis

To investigate the possible effect of electromagnetic fields on cellular viability, we evaluated the amount of metabolically active exposed and unexposed cells with MTT test. This test is especially useful for quantifying viable cells, because the incorporation of formazan dye by metabolic active cells induce their cleavage. The results of this analysis showed a statistically significant decrease ($P < 0.01$) in the total viable CCRF-CEM cells number after 24 and 48 h of exposure to 900 MHz EMF with respect to the control cells. No significant difference in cellular viability was observed between exposed and unexposed cells for shorter exposure times (2, 4, and 12 h) (Fig. 3A). Data from sham exposure, obtained by comparing cells cultured outside the TEM cell equipment or inside but with the generator turned off do not cause any alteration of viable cell number (Fig. 3B).

To assess whether the difference between control and exposed cells in cellular viability and activity depends on deregulation of cell cycle phases and/or induction of an apoptotic response, we performed FACS analysis. As it is shown in Table 1, exposure to EMFs induced a statistically significant increase of the percentage of cells

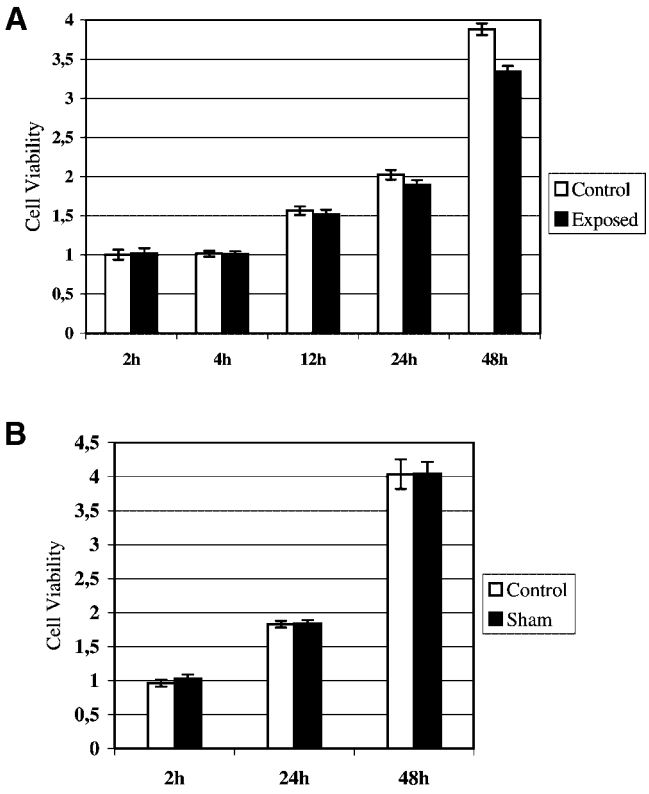

Fig. 3. **A**: Proliferation index of 900 MHz EMF exposed T-lymphoblastoid leukaemia (CCRF-CEM) cells (exp) and unexposed cells (ctrl). The proliferation index of the cells was calculated by making a ratio between the absorbance (Abs) value of the examined samples and a reference value (Abs value of cell suspension at 0 h). **B**: Proliferation index of CCRF-CEM cells cultured for 2, 24, and 48 h inside (sham) and outside the TEM cell (ctrl) without electromagnetic field emission (generator switched off). Statistical significance of the differences was evaluated by Student's *t*-test.

undergoing apoptosis after 2 h (exposed cells 18.07% vs. 3.89% control cells; $P < 0.01$). This pro-apoptotic effect of EMFs gradually decreased after 24 and 48 h of exposure notwithstanding the fact that differences between exposed and unexposed cells were still statistically significant (24 h: exposed 7.98% vs. 4.03% control, $P < 0.01$, 48 h: exposed 3.38% vs. 1.37% control; $P < 0.05$). On the other hand, statistically significant differences in the distribution of cell cycle phases were detected only after 48 h. The percentage of cells, which started DNA synthesis (S-phase) increased (39.63% in the exposed cells vs. 22.6% in the control, $P < 0.01$) while the cells which underwent growth arrest (G_0/G_1 phase) decreased (26.68% in the exposed cells vs. 40.06% in the controls, $P < 0.01$).

To confirm the flow cytometry data, which indicated a significant apoptosis induction, we performed a DNA ladder assay. Genomic DNAs extracted from exposed and control cells after 2, 24, and 48 h of culture showed a typical oligonucleosomic DNA ladder after 2 and 24 h of electromagnetic field CCRF-CEM cells exposure. No DNA ladder was detected after 48 h of EMF exposure (Fig. 4). This first series of experiments indicate that exposure to 900 MHz EMF determines a DNA damage inducing early programmed cell death in a fraction of CCRF-CEM cells. However, after longer exposure times, the surviving cells show greater viability due to higher DNA synthesis rate.

EMF biological effects on genes expression level

To investigate the activation of which genes could explain the observed effects, we evaluated the expression level of the most representative pro-apoptotic and cell cycle regulator genes in CCRF-CEM cell line at the end of various 900 MHz EMF exposure times. Data are shown in Figure 5 where we report the results of Western blot analysis for both control and exposed cells. p53 expression level was higher in exposed cells compared to the control at 2, 4, and 12 h and gradually decreased to reach basal expression level at 24 and 48 h. p21/WAF1 showed a similar trend but the increase of its level was shifted by 2 h with respect to p53 and maintained at steady level for the next 12 and 24 h to decrease at 48 h. As regard to the expression level of pro-apoptotic gene *bax*, we found a strong over-expression at 2 and 4 h followed by its gradual down-regulation. On the other hand, the Bcl-2 pro-survival oncoprotein, which antagonizes bax function, increased its expression starting from 4 h and kept steady high levels for 48 h.

The expression level of pRb/p105, which negatively controls cell proliferation, progressively increased starting from 2 h, with highest expression at 48 h, in electromagnetic field exposed cells with respect to control cells. E2F1 level increased after 2 h exposure to 900 MHz EMF and was maintained higher until 12 h while a down-regulation of E2F1 expression started at 24 h. On the other hand, in control cells E2F1 expression level was always lower with respect to exposed cells. As regard the p73 expression level, a pattern similar to that detected for p53 has been observed. In fact its level was higher in exposed cells compared to the control at 2, 4, and 12 h and gradually decreased at 24 and 48 h.

TABLE 1. FACS analysis of T-lymphoblastoid leukemia (CCRF-CEM) cells unexposed (control) and exposed to 900 MHz electromagnetic field at 2, 24, and 48 h*

Time (h)		G_0/G_1	S	G_2/M	Apoptosis
2	Control	45.75	35.55	13.42	3.89
	900 MHz EMF exposure	38.5	32.38	9.13	18.07 ($P < 0.01$)
24	Control	56.31	21.95	13.79	4.03
	900 MHz EMF exposure	54.82	22.15	11.09	7.98 ($P < 0.05$)
48	Control	40.05	22.60	33.97	1.37
	900 MHz EMF exposure	26.68($P < 0.01$)	39.83 ($P < 0.01$)	27.62	3.38 ($P < 0.05$)

*The values represent the percentage of cells in the different cell cycle phases. The underlined values are statistically significant (Student's *t*-test).

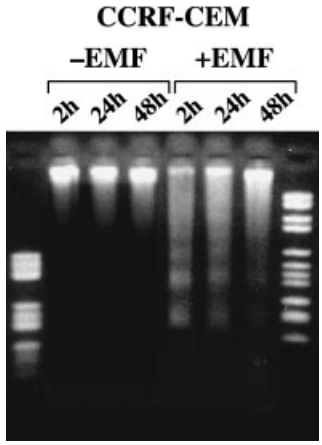

Fig. 4. DNA ladder of unexposed (−EMF) and exposed (+EMF) CCRF-CEM cells after 2, 24, and 48 h of culture.

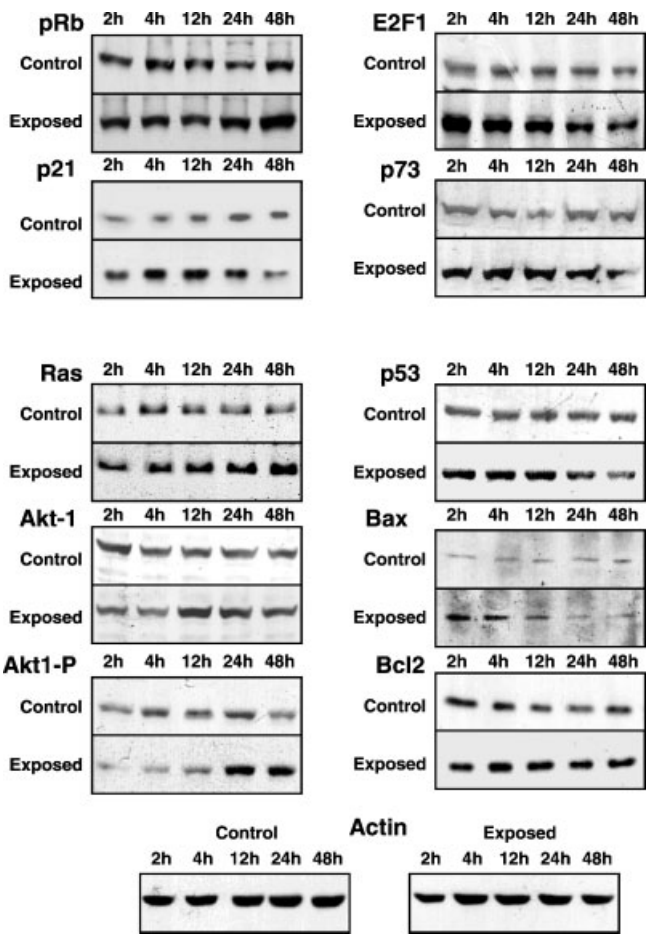

Fig. 5. Western blot analysis of genes involved in cell cycle control (pRb/p105, p21/Waf1), p53-dependent (p53, bax, bcl2), and p53-independent (E2F1, p73) apoptosis and in pro-survival signal (Ras, Akt1, Akt1-P). Forty micrograms of whole cell lysate at the various times after 900 MHz EMF exposure (exposed) and of unexposed cells (control) were electrophoretic fractionated. Western blots were normalized by using α-actin antibody.

These data indicate that the E2F1-p73 dependent pro-apoptotic pathway is also triggered at first and thereafter silenced.

To evaluate the effect of 900 MHz EMF on the expression of proteins involved in extra-cellular pro-survival signaling, we investigated the expression of the two proto-oncogenes, Ras and Akt1. Our data evidenced a quick (2 h) and progressive increment of Ras protein until 48 h in the exposed with respect to the control cells, which showed a lower expression level for all the time. On the other hand, the expression level of the Akt1 inactive form increased 12 h after EMF exposure and was kept high until 48 h while an increase of the phosphorylated-Akt1 active form (Akt1-P) was evident at 24 h and maintained high until 48 h only in exposed cells. These results show that at longer exposure times, when both p53-dependent and -independent pathways are no more effective, pro-survival signals are maximally operative.

DISCUSSION

High-frequency EMFs are a very important part of the electromagnetic spectrum and the mean level of environment emission has progressively increased in developed countries. A positive correlation between high-frequency EMFs exposure and tumorigenesis is suggested by epidemiological studies carried out in highly exposed subjects (Milham, 1985; Szmigielski et al., 1988; Goldsmith, 1995; Szmigielski, 1996; Michelozzi et al., 2002). These results are supported by a few in vitro and in vivo data showing that high-frequency EMFs can induce DNA breaks in cells (Sarkar et al., 1994; Lai and Singh, 1995, 1996; Adey, 1997; Malyapa et al., 1998; Phillips et al., 1998), chromosome aberration (Garaj-Vhrovac et al., 1990; Maes et al., 1993, 1995), changes in cell proliferation (Kwee and Raskmark, 1999; Velizarov et al., 1999) as well as activation of oncogenes transcription (Ivaschuk et al., 1997; Goswami et al., 1999).

Despite the fact that these in vivo and in vitro experiments suggest a possible damaging effect of electromagnetic field, other in vivo and in vitro studies suggest opposite points of view.

One of the controversial aspects is whether the biological changes can be induced by high-frequency EMFs through thermal or a-thermal phenomena. Some studies suggested the possibility that EMFs could affect biological systems by transferring thermal energy to particles of biological material, which possess own average thermal kinetic energy, depositing enough energy to alter some structure significantly (Moulder et al., 1999). On the other hand, other studies have established, both in vitro and in vivo, that these biochemical and molecular effects on cells may be independent from thermal phenomena produced by field exposure (Litovitz et al., 1990; Adey, 1993; Astumian et al., 1995; Barnes, 1996). The fact that EMF can produce thermal phenomena does depend on the grade of power density field administered. For this reason, in order to avoid thermal effect on our experimental setting we exposed the cells to unmodulated 900 MHz EMF with low power density field.

Studies on genotoxic potential effects of high-frequency EMFs have been done in cell culture and

animals. While some studies have not revealed significant genotoxicity (Leonard et al., 1983; Lloyd et al., 1986; Saunders et al., 1988), other studies have reported positive effects (Garaj-Vhrovac et al., 1990; Maes et al., 1993, 1995; Lai and Singh, 1995, 1996; Adey, 1997; Phillips et al., 1998). These divergent results mostly derive from non-homogeneous experimental design. In fact, in vitro and in vivo high-frequency EMF studies often differ in experimental conditions with respect to time and type of exposure (modulated or unmodulated field and power) as well as most epidemiological studies lack of systematic exposure measurements for individuals (Moulder et al., 1999).

We investigated the effects of unmodulated 900 MHz at low power density EMF to affect cellular systems by inducing both genotoxic damage and changes in gene expression levels.

We first assessed that, in our experimental conditions, unmodulated 900 MHz EMF with 1 mW input power produced the 3.5 mW/kg specific absorption value (SAR) for each cell without any relevant thermal effect. On the other hand, epidemiological and experimental data suggest that leukemic cells could be particularly susceptible to high-frequency EMFs exposure. Therefore, we investigated the effects of unmodulated 900 MHz EMF on the survival chance of human CCRF-CEM cell line, as a function of exposure time. Normal cell growth rate is the result of a balance between pro-apoptotic and pro-survival signals and any unbalance in this equilibrium predisposes to cellular transformation and, when the cell self-defense mechanisms fail, to cancer. Consequently, we focused our analysis on cellular viability and signal pathways involved in cell cycle control and apoptosis. A significant decrease in CCRF-CEM cell viability became detectable after 24–48 h of continuous 900 MHz EMF exposure. This decreased viability was the mere result of the effect of unmodulated electromagnetic field on cultured cells and was not dependent on TEM cell itself since the sham exposure did not evidence any changes in cell viability index in the absence of electromagnetic field. However, already from the first 2 h of 900 MHz exposure, DNA ladder and FACS analysis evidenced the presence of numerous apoptotic cells. During apoptosis, cellular endonucleases cleave genomic DNA between nucleosomes, producing fragments whose lengths vary by multiples of 180–200 bp (Arends and Wyllie, 1991; Enari et al., 1998). When genomic DNA extracted from apoptotic cells is resolved using agarose gel electrophoresis, these DNA fragments appear as a nucleosomal ladder, a widely recognized hallmark of apoptosis (Compton, 1992). As apoptosis is normally a protective mechanism removing DNA damaged cells, our data suggest that unmodulated EMF might act as a genotoxic agent quickly inducing DNA damage.

DNA fragmentation represents a cell signal for activation of growth arrest and pro-apoptotic genes. It is well known that the apoptotic machinery is engaged only when survival signals are withdrawn and death signals are amplified. To characterize the expression profiles of genes involved in both growth arrest and p53-dependent and -independent apoptosis, we performed Western blot analysis in CCRF-CEM cells exposed to 900 MHz EMF and compared the results with the controls at various exposure times. It is well known that there are multiple interactions between the retinoblastoma family protein pathway, whose main function is to control G_1 to S-phase progression, and p53-dependent and -independent pro-apoptotic pathways, which guard against genomic instability by inducing both arrest of the cell cycle and apoptosis (Hsieh et al., 1999). Moreover, it has been recently shown that E2F1, the main target pRb/p105 growth suppressive function, plays a dual role by directly influencing p53-dependent and -independent apoptosis execution (Johnson and Schneider-Broussard, 1998; Hsieh et al., 1999). Namely, E2F1 has been shown to induce accumulation of p53 (Hiebert et al., 1995; Kowalik et al., 1998) and transcription of p73 (Irwin et al., 2000; Stiewe and Putzer, 2000; La Sala et al., 2003). In our experimental model, an effect on growth arrest was reflected by the up-regulation of both pRb/p105 and p21/WAF1 while the induction of an apoptotic response was evidenced by the early activation of p53, bax, E2F1 and p73. Activation of the apoptotic signals was maintained for the first 24 h when it prevailed over survival signals in accordance to what observed by DNA ladder and FACS analysis.

In the face of accumulating genomic damage, the cell might also undergo an activation event that provides an increased but inappropriate level of survival signaling, which allows it to evade subsequent attempts to autodestruction. Actually, following 900 MHz EMF exposure we observed an activation of bcl-2 pro-survival oncoprotein, which antagonizes bax pro-apototic protein, as well as of both Ras and its down-stream partner, Akt1, delayed with respect to the pro-apoptotic effect. The ability of high-frequency EMFs to induce Ras activation suggests that they actually work as a stress signal. Our data suggest that the activation of proto-oncogenes such as Ras and Akt1 may provide an increase in survival potential sufficient to speed up-replication of cells with carcinogenic damage (Chin et al., 1999; Datta et al., 1999). In CCRF-CEM Ras-dependent pro-survival signals counteracted the pro-apoptotic ones only after continuous 24–48 h exposure to 900 MHz EMF as it is evidenced by the prevalence of S-phase cells observed by FACS analysis. It is known that Ras protects cells from apoptosis by activating Akt1 via PI3-kinase. Activated Akt1 (Akt1-P) is a key component of cell survival since it promotes a decrease in pro-apoptotic genes transcription and is implicated in the negative regulation of pRb/p105 functions (Kennedy et al., 1997; Downward, 1998; Du and Montminy, 1998; Rytomaa et al., 2000; Macaluso et al., 2002). We found that phosphorylated Akt1 is up-regulated after 24 h in concomitance with p53 and bax down-regulation. Collectively these data indicate that the cells initially respond to genotoxic damage induced by EMF exposure by inducing cell cycle arrest and apoptosis while at longer exposure times the activation of the p53-dependent and-independent pro-apoptotic pathway is no more effective. Therefore, notwithstanding the fact that continuous exposure of tumor cells to 900 MHz EMF induces a reduction in the number of viable cells, the survivor cells show an increase in DNA synthesis rate sustained by an activation of pro-survival signals and a consequent silencing of pro-apoptotic ones. Prevented programmed cell death due to constant signals promoting survival is critical for tumor progression and leads to metastasis formation (Chin et al., 1999;

Wong et al., 2001). Therefore, overall our data strongly support the hypothesis that high frequency EMFs exposure leads cancer cells to acquire a greater survival chance, a phenomenon linked to tumor aggressiveness.

Experiments are in progress to verify whether exposure to high frequency EMFs can promote as well the tumor transformation of normal cells.

ACKNOWLEDGMENTS

The authors thank Dr. Angelico Bedini, Claudia Gilberti and Raffaele Palomba of ISPESL institute for technical support on dosimetry controls.

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